Evaluation of bias associated with high multiplex target-specific pre-amplification

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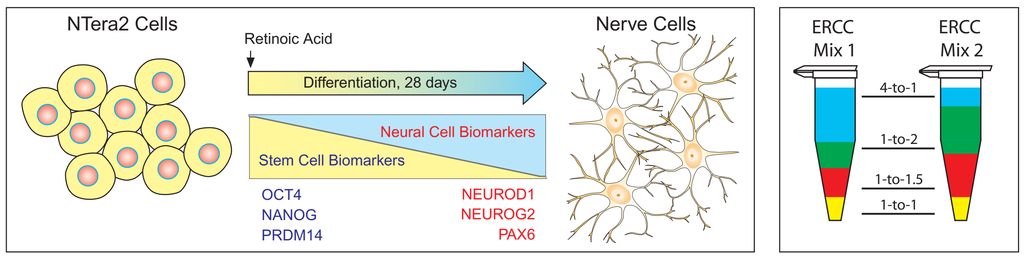
Background

Abstract

We developed a novel PCR-based pre-amplification (PreAmp) technology that can increase the abundance of over 350 target genes one million-fold. To assess bias introduced by PreAmp we utilized ERCC RNA reference standards, a model system that quantifies measurement error in RNA analysis workflows. We assessed three types of bias: amplification bias, dynamic range bias and fold-change bias. We show that PreAmp does not introduce significant amplification and fold-change bias, even under high multiplex and high amplification conditions. We do detect dynamic range bias if a target gene is highly abundant and PreAmp occurred for 16 or more PCR cycles; however, this type of bias is easily corrected. To validate PreAmp performance in a gene expression profiling experiment, we analyzed a panel of genes that are regulated during differentiation using the NTera2 stem cell model system. We find that results generated using PreAmp are statistically equivalent to results obtained using standard qPCR without the pre-amplification step. Importantly, PreAmp maintains patterns of gene expression changes across samples; the same biological insights would be derived from a PreAmp experiment and a standard gene expression profiling experiment. Our PreAmp technology can thus facilitate accurate analysis of extremely limited samples in gene expression profiling experiments.

Model systems utilized



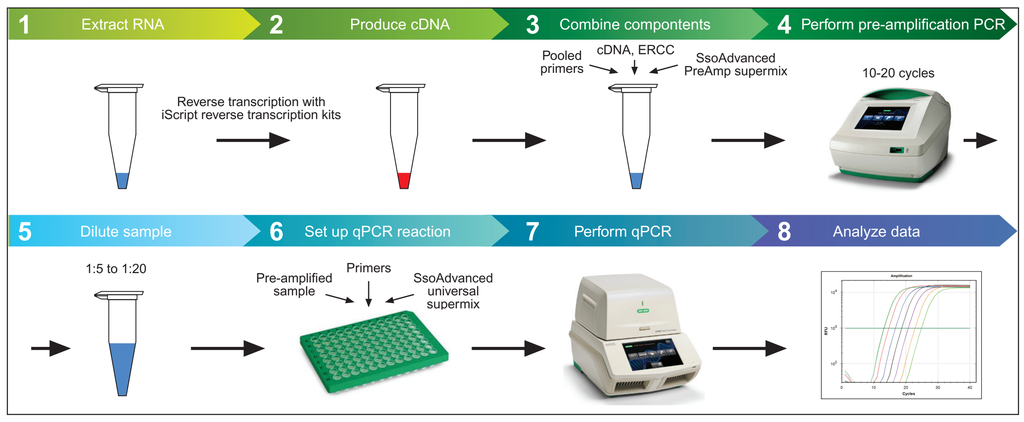
Model system of stem cell differentiation

NTera2 cells (NT2) are a model system of human stem cell behavior. When treated with retinoic acid (RA), NT2 differentiate into neurons. During differentiation the expression of stem cell biomarkers decrease and the expression of neural biomarkers increase (1). We analyzed RNA isolated from NT2 cells treated with RA for 0-7 days with a SsoAdvanced PreAmp workflow. ERCC reference standards were spiked into the RNA samples as an internal control.

ERCC model system to assess RNA quantification bias

To assess bias introduced by SsoAdvanced PreAmp we utilized ERCC RNA reference standards, a RNA measurement model system developed by the National Institute of Standards and Technology and sold by Life Technologies. The ERCC standards are mixtures of 92 synthetic RNAs that are spiked into RNA samples and processed and quantified along with the natural RNAs. The amount of each ERCC RNA in a mixture is precisely defined; the performance of an RNA quantification workflow is determined by comparing the measured amount with the actual, defined amount of each ERCC control RNA (2).

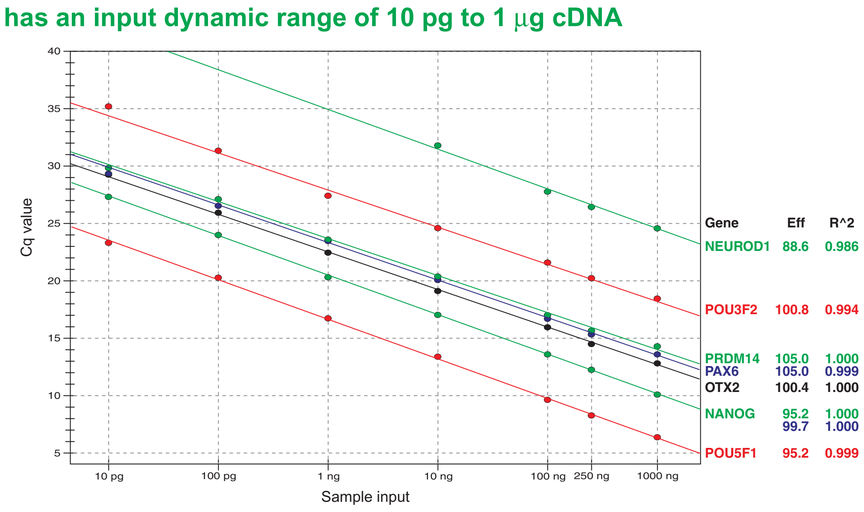
Pre-amplification workflow



SsoAdvanced PreAmp performance

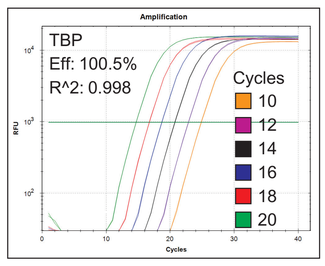
SsoAdvanced PreAmp has an input dynamic range of 10 pg to 1 µg cDNA

PreAmp was performed on 10 pg to 1 µg cDNA. Selected target genes with varied expression levels were analyzed by qPCR. The results demonstrate that SsoAdvanced PreAmp has the capacity to analyze samples with a 6-log input variation range (10 pg to 1 µg) and faithfully maintain the relative quantity of gene targets over a wide range of target gene expression levels (e.g. a span of 18 Cq from POU5F1 to NEUROD1).



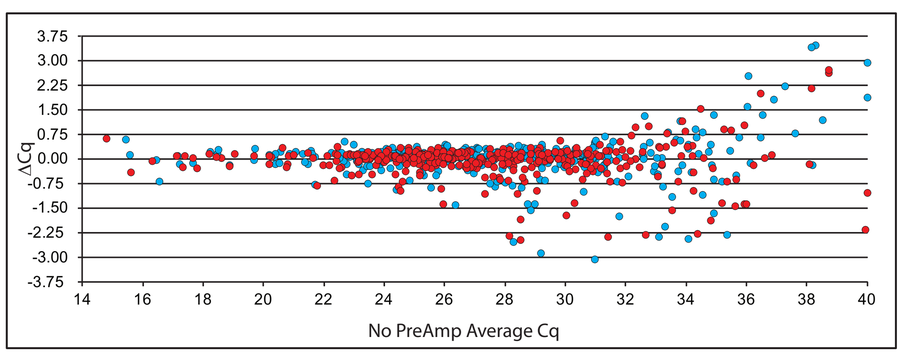
SsoAdvanced PreAmp is efficient for up to 20 PreAmp cycles

PreAmp was performed on 10 ng cDNA for 10-20 PreAmp cycles. qPCR traces for TBP are shown. Efficiency and R^2 values show that SsoAdvanced PreAmp maintains exponential amplification for at least 20 PreAmp cycles.



Amplification bias

SsoAdvanced PreAmp has minimal amplification bias

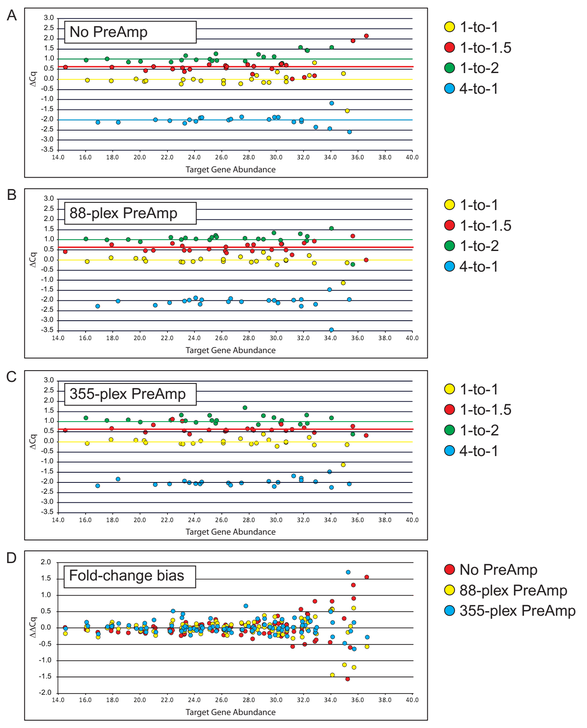


ERCC spike-in mixture 1 was added to NT2 cDNA derived from untreated cells (red circles), ERCC spike-in mixture 2 was added to NT2 cDNA derived from cells treated with retinoic acid for 7 days (blue circles); the samples were amplified in a 355-plex PreAmp reaction for 14 PCR cycles. All 355 targets were quantified by qPCR; amplification bias was determined and plotted against target gene expression. Amplification bias was calculated as (Cq Target (no-PreAmp) – Cq Target (PreAmp) – 3.03) where 3.03 reflects the theoretical ΔCq between the no-PreAmp and PreAmp target gene Cq values if PreAmp is 100% efficient.

Amplification bias reflects PreAmp PCR efficiency and is a commonly used parameter to measure PreAmp performance. Specifically, amplification bias measures whether each target is amplified at 100% efficiency throughout the PreAmp PCR cycling. We find that our SsoAdvanced PreAmp performs very well on this metric, even under high multiplex conditions.

Fold-change bias

Fold-change bias determined by ERCC RNA reference standards



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		Target gene expression (Cq) <30	30-32.5	>32.5
No PreAmp	Average fold change bias (Cq)	0.093	0.265	0.804
	Average fold change bias (percent)	7%	20%	75%
88-plex PreAmp	Average fold change bias (Cq)	0.094	0.162	0.578
	Average fold change bias (percent)	7%	12%	49%
355-plex PreAmp	Average fold change bias (Cq)	0.108	0.119	0.380
	Average fold change bias (percent)	8%	9%	30%
	Estimated fold-change limits-of-detection	2-fold	4-fold	8-fold

ERCC spike in-mixtures were added to NT2 cDNA samples and pre-amplified; ERCC targets were quantified by qPCR. Fold-change measurements for each ERCC target are plotted against target abundance. Results show the measured difference, between samples, of the amount of each ERCC target (colored circles) and the actual difference that should be detected (colored lines). (A) Analysis of samples that were not pre-amplified. (B) Analysis of samples in which only the 88 ERCC targets were pre-amplified. (C) Analysis of samples in which 355 targets were pre-amplified. (D) Overall fold-change bias for 88 ERCC targets. No PreAmp samples (red circles), 88-plex PreAmp samples (yellow circles), 355-plex PreAmp samples (blue circles). (E) Table listing average fold-change bias in groups based on target gene abundance; the fold-change limits of detection are conservatively estimated based upon the 95% confidence intervals of fold-change bias.

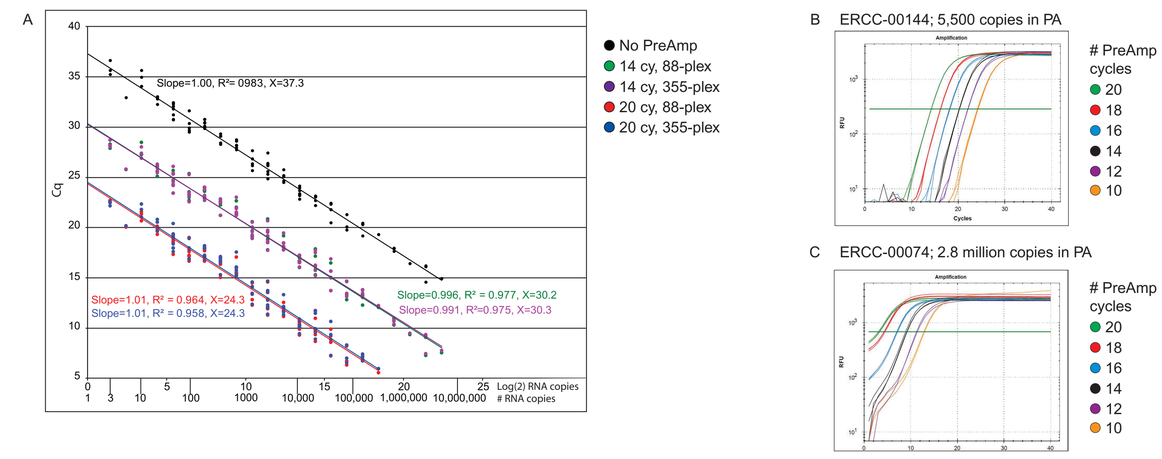
Quantification bias of natural target genes

cDNA derived from NT2 cells treated with RA for 0-7 days were pre-amplified and used to quantify native target genes by qPCR; 100 pg, 1 ng and 10 ng cDNA was used in the PreAmp reactions. 1 µg cDNA that was not pre-amplified was also used to quantify target gene expression by standard qPCR (10 ng per target). The level of expression of each target gene was determined and expressed as "RNA copies per cell" after normalizing with TBP and assuming that there are 10 TBP copies per cell (3). The experiment was performed three times. Error bars represent standard deviation. Data for selected target genes are shown.

We find that, relative to standard gene expression analysis, PreAmp does not cause statistically significant fold-change bias in the gene expression profiles of all target genes analyzed, even when 10,000-fold less cDNA is used. These findings demonstrate that SsoAdvanced PreAmp provides accurate and reproducible quantification of both stem cell and neural cell biomarkers and does not bias gene expression profiling results. Importantly, we also show that SsoAdvanced PreAmp faithfully maintains patterns of relative gene expression changes; the same insights and conclusions would be drawn from a SsoAdvanced PreAmp-based experiment and a standard gene expression profiling experiment.

Dynamic range bias

Dynamic range bias with a high number of PreAmp cycles



ERCC mixtures were added to NT2 cDNA, pre-amplified for 10 to 20 PCR cycles and quantified by qPCR. (A) The measured amount of each ERCC RNA target is plotted as a function of actual RNA abundance. All ERCC RNA targets can be detected in the no PreAmp sample and in the samples processed for 14 PreAmp cycles. For samples processed for 20 PreAmp cycles, quantification of targets in the 1 million copy range, have a Cq value less than 5 and are of unreliable value. (B) qPCR results for ERCC-00144, which contained 5,513 copies in the PreAmp reaction, show good results over the entire amplification range. (C) qPCR results for ERCC-00074, which contained 2,822,879 copies in the PreAmp reaction, show poor results when amplified for 18 or 20 PCR cycles.

Dynamic range bias measures the ability to accurately quantify target genes over a wide range of abundance. Our standard, no PreAmp gene expression profiling workflow can quantify the entire 6-log dynamic range of the ERCC controls. Pre-amplification for up to 16 PCR cycles can also accurately quantify the entire ERCC assay set and, thus, does not introduce dynamic range bias. However, in instances of high target gene expression coupled with a high number of PreAmp cycles (e.g. 20 cycles), target gene quantification may be compromised. We believe that dynamic range bias will not be an issue in most PreAmp experiments. Dynamic range bias is apparent in the qPCR traces, and can be alleviated through sample dilution or use of fewer PreAmp cycles.

Summary and conclusions

We have developed a new reagent, SsoAdvanced PreAmp Supermix, which allows for accurate quantification of over 350 target genes from very small samples. We envision that this product can benefit researchers who work with limited or rare samples, and can lead to advances in the single cell analysis field.

References

1. Pleasure, S.J., and V.M. Lee. 1993. NTera 2 cells: a human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J. Neurosci. Res. 35:585–602.
2. Qu, L., A. Lemire, K. Lea, D. Batten, S. Jian Gu, P. Whitley, and K. Bramlett. 2011. Development of ERCC RNA Spike-In Control Mixes. J Biomol Tech. 22 (Suppl):S46.
3. Taniguchi, K., T. Kajiyama, and H. Kambara. 2009. Quantitative analysis of gene expression in a single cell by qPCR. Nat. Methods 6:503–506.